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Gene expression modulation of ABC transporter genes in response to permethrin in adults of the mosquito malaria vector *Anopheles stephensi*

Valentina Mastrantonio[a,1], Marco Ferrari[b,1], Sara Epis[b,e,*], Agata Negri[a], Giulia Scuccimarra[a], Matteo Montagna[c], Guido Favia[d], Daniele Porretta[a], Sandra Urbanelli[a], Claudio Bandi[b,e]

[a] *Department of Environmental Biology, Sapienza University of Rome, Via dei Sardi 70, 00185 Rome, Italy*
[b] *Department of Biosciences, University of Milan, Via Celoria 26, 20133 Milan, Italy*
[c] *Department of Agricultural and Environmental Sciences, University of Milan, Via Celoria 2, 20133 Milan, Italy*
[d] *School of Bioscience and Veterinary Medicine, University of Camerino, Via Gentile III da Varano, 62032 Camerino, Italy*
[e] *Pediatric Clinical Research Center Romeo and Enrica Invernizzi, Ospedale "Luigi Sacco", Via Giovanni Battista Grassi, 74, 20157 Milan, Italy*

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ABSTRACT

Living organisms have evolved an array of genes coding for detoxifying enzymes and efflux protein pumps, to cope with endogenous and xenobiotic toxic compounds. The study of the genes activated during toxic exposure is relevant to the area of arthropod vector control, since these genes are one of the targets upon which natural selection acts for the evolution of insecticide resistance. ATP-binding cassette (ABC) transporters participate to insecticide detoxification acting as efflux pumps, that reduce the intracellular concentration of toxic compounds, or of their metabolic derivatives. Here we analyzed the modulation of the expression of six genes coding for ABC transporters, after the exposure of adult females and males of the mosquito *Anopheles stephensi*, a major malaria vector in Asia, to permethrin. Male and female mosquitoes were exposed to insecticide for one hour, then the expression profiles of the ABC transporter genes *Anst*ABCB2, *Anst*ABCB3, *Anst*ABCB4, *Anst*ABCBmember6, *Anst*ABCC11, and *Anst*ABCG4 were analysed after one and 24 h. Our results showed that three genes (*Anst*ABCB2, *Anst*ABCBmember6, *Anst*ABCG4) were up-regulated in both sexes; two of these (*Anst*ABCBmember6 and *Anst*ABCG4) have previously been shown to be up-regulated also in larval stages of *An. stephensi*, supporting a role for these genes in permethrin defence in larvae as well as in adults. Finally, the same ABC transporter genes were activated both in females and males; however, the timing of gene induction was different, with a prompter induction in females than in males.

1. Introduction

Arthropods defend themselves from physiological metabolites and from both natural (e.g. plant and microbial toxins) and anthropic exogenous toxicants (e.g. organic pollutants and pesticides) by detoxifying protein systems, encoded by an array of genes and gene families; these systems, composed by detoxifying enzymes and efflux pumps, transform the toxic chemicals and/or eliminate them from cells (Goldstone, 2008; Steinberg, 2012). Identifying which genes and gene-family members are activated during the detoxification response is relevant to the control of arthropod vectors, as genes coding for detoxifying proteins are one of the substrates upon which natural selection acts for the evolution of insecticide resistance. In addition, detoxifying enzymes and transporters have the potential to be exploited as targets for the development of novel insecticides (Buss and Callaghan, 2008; Le Goff et al., 2006; Steinberg, 2012).

In recent years, ATP-binding cassette (ABC) transporters, integral membrane proteins belonging to the ABC family, have attracted a great deal of attention (Dermauw and Van Leeuwen, 2014; Merzendorfer, 2014). Differently from detoxifying enzymes (e.g. cytochrome-P450, or glutathione-S transferases), ABC transporters participate to cell defence not by modifying toxic substrates, but

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* Corresponding author at: Department of Biosciences, University of Milan, Via Celoria 26, 20133 Milan, Italy.
E-mail address: sara.epis@unimi.it (S. Epis).

[1] Co-primary authorship.

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reducing their intracellular concentration, by extruding toxicants out of the cells once they have entered into them, or after that they have been modified by detoxifying enzymes (Dermauw and Van Leeuwen, 2014; Merzendorfer, 2014). Among the ABC transporter families, particularly important are the ABC transporters belonging to the ABCB (also called MDRs or P-gps), ABCC and ABCG subfamilies (Dermauw and Van Leeuwen, 2014) that are considered the "first line of cellular defence" against a wide range of xenobiotics, including insecticides (Buss and Callaghan, 2008).

As already emphasized, the interest about ABC transporters in arthropods derives on one hand from the need to understand the molecular basis of insecticide defence, on the other hand from the perspective to discover new molecular targets for novel insecticides. In addition, these transporters could be exploited to improve the efficacy of 'conventional' insecticides, thanks to the synergistic effects with ABC transporters inhibitors (Buss and Callaghan, 2008; Cafarchia et al., 2015; Dermauw and Van Leeuwen, 2014; Pohl et al., 2011; Porretta et al., 2016). Indeed, sequence-specific gene silencing by RNA interference (RNAi), in different blood-sucking insect species, has shown the possibility to specifically suppress the ABC transporter gene expression, increasing the susceptibility of tested individuals (Dalla Bona et al., 2016; Figueira-Mansur et al., 2013; Yoon et al., 2011; Zhu et al., 2013).

In mosquitoes, ABC transporters have been shown to be involved in defence against several insecticide classes (Dermauw and Van Leeuwen, 2014). Bioassays using insecticides and ABC transporter inhibitors (e.g. verapamil or cyclosporin A) have shown that insecticide toxicity increased after the inhibition of these transporters. For example, increased larval mortality was observed in larvae of susceptible populations of the mosquitoes *Aedes caspius* and *Aedes aegypti* exposed to temephos or in *Culex pipiens* larvae exposed to endosulfan, ivermectin and cypermethrin (Buss et al., 2002; Figueira-Mansur et al., 2013; Lima et al., 2014; Porretta et al., 2008). Likewise, in both *Anopheles* and *Aedes* species, up-regulation of ABC transporter genes has been observed in mosquito larvae from susceptible populations after insecticide exposure, supporting the view that ABC transporters play an important role in mosquito defence against chemicals (Epis et al., 2014a,b; Figueira-Mansur et al., 2013). For example, in *Ae. aegypti* larvae exposed to temephos, the authors observed a significant increase of the expression of the ABCB transporter *AaegP-gp*, up to eight-fold after 48 h (Figueira-Mansur et al., 2013).

Anopheles stephensi is the main malaria vector in the Middle East and South Asian regions (Sinka et al., 2010; WHO, 2016a). In this species, ABC transporters have been implicated in the defence against the pyrethroid insecticide permethrin, in larvae belonging to an insecticide-susceptible strain (Epis et al., 2014a). Furthermore, the analysis of the transcriptional response of six ABC transporter genes, identified in the larval transcriptome, showed both up- and down-regulation at different time points, which suggests the occurrence of a modulated response of ABC transporter genes during permethrin exposure.

To date, despite pyrethroids occupy a prominent position in adult control, studies focused on ABC transporters and pyrethroid insecticides in *An. stephensi* adults are limited (Vontas et al., 2007). Mosquito larvae and adults are naturally exposed to different endogeneous and xenobiotic compounds (Clements, 2000). The modulation of ABC transporter gene expression, in response to toxicants exposure, can thus be expected to differ in different life stages of the mosquito, as observed in other insect species. For example, the overexpression of an ABCG gene was observed in adults of the whitefly *Bemisia tabaci*, while no overexpression was observed in eggs and nymphae (Yang et al., 2013), which agrees with the idea that ABC transporter response to insecticides varies during insect development. On the other hand, in the salmon louse *Caligus rogercresseyi* the ABC transporter gene *Cr-Pgp* was found up-regulated in females after delthametrin exposure, but not in males, suggesting that differential responses are possible not only relative to the developmental stage, but also to sex (Valenzuela-Muñoz et al., 2014).

The aim of this work was to investigate the transcriptional response of ABC transporter genes to permethrin in adults of *An. stephensi*. Male and female mosquitoes were exposed to permethrin separately; then the expression profiles of six ABC transporter genes were determined, at two time-points after insecticide exposure (one and 24 h). Gene expression data were analysed to assess the possible occurrence of differences across time and/or between sexes, and discussed in relation with the results obtained on larvae of *An. stephensi* in previous studies (Epis et al., 2014a,b).

2. Material and methods

2.1. Mosquitoes

All mosquitoes used in this study derived from the *Anopheles stephensi* Liston colony of the insectary of the University of Camerino, Italy. Mosquitoes were reared at 28 ± 1 °C with a relative humidity of 85–90%, and a 12:12 Light-Dark photoperiod. All experiments were conducted separately on adult males and females. At this aim, the eggs were kept in spring water until hatching; larvae were fed daily with fish food (Tetra, Melle, Germany) and sex-separated as pupae; groups of 25 male and female adults were maintained in different cages and fed with a 5% sucrose solution until laboratory bioassays.

2.2. Mosquitoes exposure to permethrin

In order to analyse the expression profile of ABC transporter genes after permethrin exposure, male and female adults were treated with permethrin following the WHO tube protocol (WHO, 2016b) with some modifications. All experiments were carried out separately for male and female mosquitoes using 3–4 day-old unfed individuals, maintained at the same temperature, humidity and photoperiod conditions of the reared colony. Permethrin (PESTANAL, Sigma-Aldrich S.r.l., Milan, Italy) was diluted in acetone to have a final concentration of 0.45% that, accordingly to WHO (2016b) and our preliminary treatments, leads to about 50–80% mortality rate after 24 h. Twenty-five males or females were gently placed into separate tubes and exposed for one hour to papers (12 × 15 cm) (Whatman® No. 1, Sigma-Aldrich S.r.l., Milan, Italy) impregnated with 2 ml of permethrin solution mixed with the silicon oil non-volatile carrier (Sigma-Aldrich S.r.l., Milan, Italy), accordingly to WHO (2016b). Papers impregnated with the silicone oil carrier and acetone (i.e. without insecticide) were used as controls. Seven replicates were conducted for permethrin treatments and four for controls.

ABC transporter gene expression was analysed at two time points (one and 24 h). In a first experiment we exposed adult males and females to permethrin for one hour as described above, then the number of dead mosquitoes was recorded in both the exposure and control tubes. A mosquito was considered dead if it was immobile or unable to stand or fly in a coordinated way. The survived individuals in both the exposure and the control tubes were collected, divided into three pools of 10 individuals for controls and three for exposed individuals and stored in RNA later at −80 °C for RNA extraction and molecular analysis. In the second experiment, we exposed adult males and females to permethrin for one hour. Then, the survived individuals were transferred in recovery tubes and supplied with sucrose solution. After 24 h, the number of dead mosquitoes in the recovery tubes was recorded and the survived individuals were collected for treatment and control (three pools